Comparative Study of Four Membranes for Evaluation of New Insect/Arthropod Repellents Using *Aedes aegypti*

WATANAPORN DHERANETRA[1], KENDRA L. LAWRENCE [1], JOHN PAUL BENANTE[1], MARK A. POTTER[1], KAMALESH R. CHAUHAN[2], NAGENDRABABU BATHINI[2], CHARLES E. WHITE[3], BRYAN MOTT[4], DANIEL A. NICHOLS[4], APURBA K. BHATTACHARJEE[4], AND RAJ K. GUPTA*[5]

[1]Walter Reed Army Institute of Research, USA, [2] Agriculture Research Center Chemicals Affecting Insect Behavior Laboratory, Beltsville, USA, [3]Walter Reed Army Institute of Research, USA, [4] Walter Reed Army Institute of Research, USA,[5] Walter Reed Army Institute of Research,USA

Introduction

Repellent use is one of the most effective personal protection measures in reducing bites of blood-sucking insects/arthropods and preventing vector-borne disease transmission. Traditionally, discovery of new repellents involves initial screening of thousands of candidates using various methods with very few successes. However, despite the obvious desirability of finding an effective mosquito repellent, no ideal repellent has been identified yet [Gupta 1994]. The process is even more complicated because of the characteristics required in an ideal insect repellent such as, effective against broad spectrum of species, long duration of protection, no toxicity or side effects, resistant to abrasion, greaseless and odorless. In addition, lack of understanding of the mode of action of repellents brings further complication to the process. Thus, a search for an ideal insect/arthropod repellent continues.

Presently, the U.S. military's most effective personal protection system [Armed Forces Pest Management Board 1996; McCabe *et al* 1964] utilizes a controlled-release formulation of DEET (*N,N* -diethyl-1,3-toluamide) as a topical repellent, which defends against several types of biting arthropods [Gupta 1994]. However, DEET has many short comings. As a repellent for human use, DEET is not equally effective against all insects and arthropod disease vectors. Moreover, it has several disagreeable cosmetic effects such as unpleasant odor and an oily feel. DEET's deep skin penetration can cause drug-drug interactions that could lead to potential toxicity in children and adults when used in high concentrations [Briassoulis, *et al* 2001]. DEET is a known plasticizer that reacts with certain plastics and synthetic rubber [Gupta 1994; Skinner and Johnson 1980; Watanabe *et al* 1993]. Finally, a growing segment of the consumer population is shying away from synthetic chemicals in favor of botanical products.

We initiated a research effort to explore the feasibility of understanding the molecular mechanism of known repellents with the objective to stream line and expedite design and discovery of insect repellents using state-of-the-art technologies. We gained valuable insights on the molecular mechanism of repellents [Bhattacharjee *et al* 1999; Bhattacharjee , Gupta and Karle 2000] and our efforts resulted in the development of a three dimensional (3D) pharmacophore to predict repellent potency of new compounds using *in silico* techniques [Bhattacharjee *et al* 2005]. However, during rapid screening of new potential repellent candidates in an *in vitro* test system [Rutledge *et al* 1978] in the laboratory, we yet faced another challenge. The supplier of Baudruche membrane used in our *in vitro* test system for years decided to retire from the market and had no plans to continuously provide the membranes. Thus, in an effort to find an alternative *in vitro* membrane test system, our initial literature search narrowed the available choices in a relatively short period of time to four membranes. They were: Baudruche, Hemotek, Sausage, and a Silicone-based membrane.

In this study, we report the results of (Gupta 1994) search and selection of a new membrane for use in an *in vitro* test system, and (Armed Forces Pest Management Board. 1996) repellent potential of the 3D pharmacophore-based newly designed and synthesized ten insect/arthropod repellents.

Test Insects: We used nulliparous female *Ae. aegypti* mosquitoes (Red eye Liverpool strain) that were laboratory-reared and maintained at 28°C and 80% RH under a photoperiod of 12:12 (L:D) h using standard mosquito rearing procedures [Rueda , Rutledge and Gupta 1998 ; Gerberg ,1970] Larvae were fed a diet of ground tropical fish flakes (Tetramin Tropical Fish Flakes, Tetra Sales, Blacksburg, VA, http://www.tetra-fish.com) and adults were fed a 10% sucrose solution. We used adult female mosquitoes between 5 and 15 day old that were starved (provided only water) for 24h before testing.

Test membranes and repellent compounds: Four membranes evaluated were: Baudruche (Joseph Long Inc, NJ); Hemotek (Discovery Workshop); sausage (Devro-Edicol™ Collagen) or a silicone-based membrane. Technical grade DEET was obtained from Sigma, St. Louis, MO. Ten new candidate repellents were custom synthesized based on our earlier reported 3D pharmacophore model [Bhattacharjee *et al* 2005] at the Division of Experimental Therapeutics, Walter Reed Army Institute of Research for CIS compound database [The Chemical Information System]. The compounds were: 1 ((3-furylmethyl octyl ether); 2 (3-furylmethyl-5-hexenyl ether); 3 (3-furyl-heptyl ketone); 4 (3-furyl-(3-ethyl)pentyl ketone); 5 (3-furyl-undecyl ketone); 6 (Furan-3-carboxylic acid dodecyl ester); 7 (Furan-3-carboxylic acid

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE **2008**	2. REPORT TYPE	3. DATES COVERED **00-00-2008 to 00-00-2008**

4. TITLE AND SUBTITLE **Comparative Study of Four Membranes for Evaluation of New Insect/Arthropod Repellents Using Aedes aegypti**	5a. CONTRACT NUMBER 5b. GRANT NUMBER 5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER 5e. TASK NUMBER 5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **Walter Reed Amy Institute of Research,503 Robert Grant Avenue ,Silver Spring,MD,20910**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S) 11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

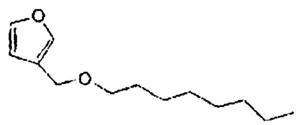
15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT **unclassified**	b. ABSTRACT **unclassified**	c. THIS PAGE **unclassified**	**Same as Report (SAR)**	**6**	

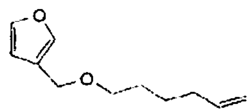
Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std Z39-18

octyl ester); 8 (Furan-3-carboxylic acid decyl ester); 9 (Furan-3-carboxylic acid 2-methyl-pentyl ester); and 10 (Furan-3-carboxylic acid 2-ethyl-hexyl ester) (Chart 1).

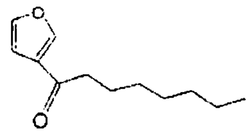
Chart 1 Structures of the pharmacophore driven custom synthesized compounds



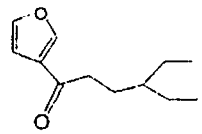
1 (3-furylmethyl octyl ether)



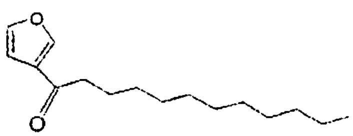
2 (3-furylmethyl-5-hexenyl ether)



3 (3-furyl-heptyl ketone)

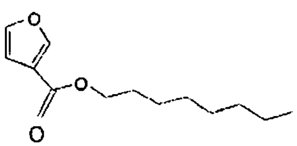


4 (3-furyl-(3-ethyl)pentyl ketone)

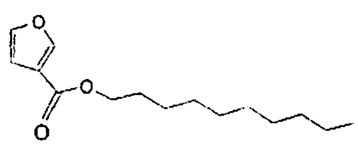


5 (3-furyl-undecyl ketone)

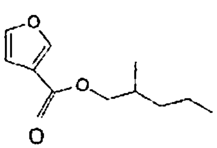
6 (Furan-3-carboxylic acid dodecyl ester)



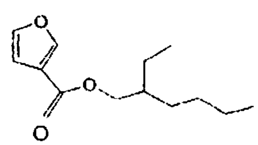
7 (Furan-3-carboxylic acid octyl ester)



8 (Furan-3-carboxylic acid decyl ester)

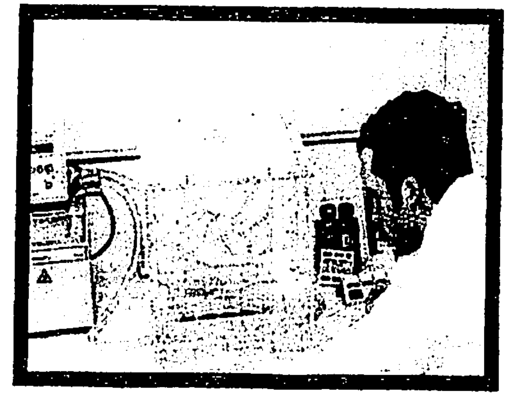


9 (Furan-3-carboxylic acid 2-methyl-pentyl ester)



10 (Furan-3-carboxylic acid 2-ethyl-hexyl ester)

Repellent test system: A modified *in vitro* test system was used to evaluate the potential membrane alternatives as shown in Fig. 1.



The test system consists of a mosquito blood feeder, a constant temperature water circulator to warm the blood, and the test cage. The blood feeder had five circular blood reservoirs, each of which was filled with outdated human blood obtained from the Walter Reed Army Medical Center Blood Bank, Washington, DC. The blood was replenished with 5 mM adenosine triphosphate (ATP), without which the mosquitoes will not feed freely [Rutledge *et al* 1976]. The blood was maintained at 37 °C with water from the constant temperature water circulator (Lauda E100, Wobser GMBH & Co., Konigshofell, Germany). The blood-filled reservoirs were covered with membrane using high-vacuum grease (Dow Corning, Midland, MI). DEET or the test materials were applied on the membrane at random including the control. The mosquitoes to be tested were given access to the blood reservoirs on a "free choice" basis by means of a sliding door in the bottom of the test cage. The number of mosquitoes feeding on each well was counted and noted at 2 minute intervals for 20 minutes using the total number of feeding mosquitoes. Only the minimum number of necessary replications were performed to ensure reproducibility of the tests results.

Data analysis: Data was analyzed using the Goldstein's free choice procedure employing an instantaneous sampling method [Goldstein 1964]. A logprobit analysis (LogPro) was carried out to calculate ED_{50} and ED_{95} values for DEET and each insect repellent compound, and then compared using one-way ANOVA.

Results and Discussions

Test membranes: Female *Ae. aegypti* fed readily on all four membranes. The ED_{50} and ED_{95} values of DEET-treated membranes observed during the repellency comparison study are presented in Table 1. No significant statistical differences were found among the four membranes when compared using a one way analysis of variance (ANOVA) as shown in Table 1. Based upon our

observation, any one of the three experimental membranes (Hemotek, sausage, or silicone-based) could be used as an alternative to Baudruche. We chose sausage membrane as a substitute for Baudruche because of its low cost, availability and for easy application of repellent test materials. The ten new repellent test compounds were evaluated using the newly selected sausage membrane.

Table 1 *In vitro* repellency of DEET using four membranes against *Ae. aegypti*

Membrane type	ED_{50}	ED_{95}	R^2	G	Slope
Baudruche	0.021 (0.019 to 0.023)	0.161 (0.148 to 0.176)	0.999	0.004	-1.861950
Hemotek	0.025 (0.008 to 0.040)	0.236 (0.128 to 1.494)	0.969	0.301	-1.686893
Sausage	0.027 (0.017 to 0.036)	0.184 (0.130 to 0.338)	0.990	0.097	-1.969844
Silicone	0.030 (0.019 to 0.040)	0.119 (0.088 to 0.193)	0.988	0.113	-2.761482

(ED_{50} = the concentration required to repel 50% of the mosquito population;
ED_{95} = the concentration required to repel 95% of the mosquito population)

Repellent potential of compounds: Table 2 shows the ED_{50} and ED_{95} values of the ten experimental repellent compounds. Five of the ten candidate repellent compounds: 3 (3-furyl-heptyl ketone); 4 (3-furyl-(3-ethyl)pentyl ketone); 6 (Furan-3-carboxylic acid dodecyl ester); 7 (Furan-3-carboxylic acid octyl ester); and 10 (Furan-3-carboxylic acid 2-ethyl-hexyl ester) exhibited superior repellency as compared to DEET. Candidate repellent compound 3 (3-furyl-heptyl ketone) provided the best repellent activity using the *in vitro* test system as shown in Table 2. However, there was no significant difference observed among the rest of the experimental repellent compounds. The observed repellency potential of three compounds: 2 (3-furylmethyl-5-hexenyl ether); 5 (3-furyl-undecyl ketone); and 9 (Furan-3-carboxylic acid 2-methyl-pentyl ester) was less than DEET whereas the remaining compounds exhibited either better or equal repellency to DEET.

Table 2 *In vitro* repellency of candidate insect repellents against *Ae. aegypti* (mg/cm^2)

Candidate repellents	ED_{50}	ED_{95}	R^2	G
DEET	0.017 ± 0.027-0.036	0.129± 0.184-0.337	0.989679	0.096535
1	0.035 ± 0.047-0.060	0.203± 0.326-0.775	0.989289	0.100223
2	0.041 ± 0.063-0.10	0.197± 0.415-3.42	0.973207	0.254835
3	0.000034 ± 0.001	0.083 ± 0.131-0.400	0.970643	0.279958
4	0.021 ± 0.032-0.042	0.082 ± 0.109-0.170	0.988659	0.106178
5	0.046 ± 0.055-0.067	0.390 ± 0.639-1.39	0.994010	0.055781
6	0.019 ± 0.032-0.043	0.082 ± 0.113-0.195	0.985234	0.138731
7	0.012 ± 0.023-0.033	0.080 ± 0.110-0.187	0.985092	0.140082
8	0.029 ± 0.034-0.038	0.240 ± 0.303-0.410	0.997835	0.020083
9	0.047 ± 0.052-0.057	0.329 ± 0.411-0.541	0.998568	0.013275
10	0.023 ± 0.0502-0.092	0.090 ± 0.164-1.49	0.955515	0.430943

(Contd.)

ED_{50} = the concentration required to repel 50% of the mosquito population;
ED_{95} = concentration required to repel 95% of the mosquito population

The 3D Pharmacophore model: The pharmacophore and its mapping on DEET are shown in Figure 2. Additionally, the pharmacophore mappings onto the ten new candidate repellents are shown in Figures 3 and 4. The successful mapping of all the features (one hydrogen-bond acceptor site, two aliphatic sites and one aromatic ring site) of the pharmacophore in the above custom designed compounds thus validated the potential of our model. In addition, the observed ED_{50} values indicate the predictive power of the pharmacophore for design and selection of new repellent compounds.

***Fig. 2** Pharmacophore (left) and its mapping on DEET (right)

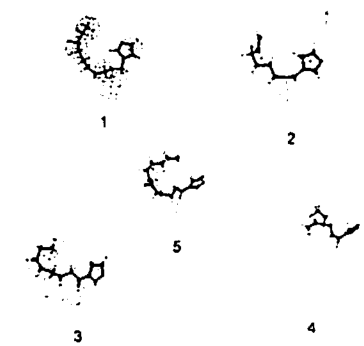


***Fig. 3** Mapping of the pharmacophore on candidate new insect repellents (1-5)

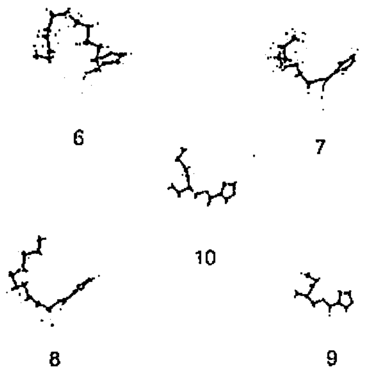


*Fig. 4 Mapping of the pharmacophore on candidate new insect repellents (6-10)

In conclusion, the present study to search a new membrane for *in vitro* repellent test system led us to successfully use sausage membrane with no significant difference in the ED_{50} and ED_{95} values obtained with Baudruche membrane. Our choice for the sausage membrane as a substitute for Baudruche was based upon its low cost, quick availability and easy application of repellent test materials. In addition, this study also validated the predictive power of our earlier reported 3D pharmacophore model by observed repellency potential of newly designed and synthesized insect/arthropod repellents compounds in an *in vitro* test system in the laboratory.

Acknowledgements

We would like to thank Nancy L. McLean-Cooper (Department of Entomology, Walter Reed Army Institute of Research) for her technical support and in rearing the mosquitoes.

References

1. Armed Forces Pest Management Board. 1996. Personal protective techniques against insects and other arthropods of military significance. Technical Guide No.36, Defense Pest Management Information Analysis Center, Forest Glen Section Walter Reed Army Medical Center, Washington, DC.
2. Bhattacharjee AK, Dheranetra W, Nichols DA, Gupta RK. 3D Pharmacophore model for insect repellent activity and discovery of new repellent candidates. *QSAR Comb. Sci.* 2005; 24: 593-602.
3. Bhattacharjee AK, Gupta RK, Ma D, Karle JM. Molecular similarity analysis between insect juvenile hormone and N, N-diethyl-m-toluamide (DEET) analogs may aid design of novel insect repellents. *J. Mol. Recogni.* 2000; 13: 213-220.
4. Briassoulis G, Narlioglou M, Hatzis T. Toxic encephalopathy associated with use of DEET insect repellents: a case analysis of its toxicity in children. *Human and Experimental Toxicology.* 2001; 20: 8-10.
5. Gerberg EL. Manual for mosquito rearing and experimental techniques. *J. Am. Mosq. Control* Assoc. 1970; 5: 109.
6. Goldstein A. Biostatistics: An Introductory Text, New York: Macmillan, (1964).
7. Gupta RK, Rutledge LC. Role of repellents in vector control and disease prevention. *Am. J. Trop. Med. Hyg.* 50 (suppl).1994; 82-86.
8. Ma D, Bhattacharjee AK, Gupta RK, Karle JM. Predicting mosquito repellent potency of N,N-diethyl-m-toluamide (DEET) analogs from molecular electronic properties. *Am. J. Trop. Med. Hyg.* 1999; 60: 1-6.
9. McCabe ET, Barthel WF, Gertler SI, Hall SA. Insect repellents. III. N, N-diethylamides, *J. Org. Chem.* 1954; 19: 493-498.
10. Rueda LM, Rutledge LC, Gupta RK. Effect of skin abrasions on the efficacy of the repellent deet against *Aedes aegypti. J. Am. Mosq. Cont. Assoc.* 1998; 14: 178-182.
11. Rutledge LC, Moussa MA, Lowe CA, Sofield RK. Comparative sensitivity of mosquito species and strains to the repellent diethyl toluamide. *J. Med. Entomol.*1978; 14: 536-541.
12. Rutledge, LC, Moussa MA, Belletti CJ. An *in vitro* blood feeding system for quantitative testing of mosquito repellents. *Mosq. News.* 1976; 24: 407-408.
13. Skinner WA, Johnson HL. The design of insect repellents. *Drug Design.* 1980; 10: 277-302.
14. The Chemical Information System, Division of Experimental Therapeutics, Walter Reed Army Institute of Research, 503 Robert Grant Avenue, Silver Spring, MD 20910-7500, U.S.A.
15. Watanabe K, Shono Y, Kakimizu A, Okada A, Matsuo N, Satoh A, Nishimura H. New mosquito repellent from *Eucalyptus camaldulensis. J. Agri Food Chem.* (1993); 41: 2164-2166.

Tata McGraw-Hill

Published by Tata McGraw-Hill Publishing Company Limited,
7 West Patel Nagar, New Delhi 110 008.

ISBN (13): 978-0-07-025177-9
ISBN (10): 0-07-025177-0

Managing Director: *Ajay Shukla*
Head—Professional and Healthcare: *Roystan La 'Porte*
Publishing Manager—*Professional: R Chandra Sekhar*
Junior Sponsoring Editor: *Nimisha Goswami*
Manager—Sales & Marketing: *S Girish*
Controller—Production: *Rajender P Ghansela*
Asst. General Manager—Production: *B L Dogra*

Typeset at Script Makers, 19, A1-B, DDA Market, Paschim Vihar, New Delhi 110 063, and printed at Sai Printo Pack Pvt. Ltd., A-102/4, Okhla Industrial Area, Phase II, New Delhi 110 020.

Cover Design: Kapil Gupta, Delhi

Cover Printer: Sai Pinto Pack

RLDLXRRXXXRZZD

The McGraw·Hill Companies

Arthropod Borne Viral Infections— Current Status and Research

Editors

D. Raghunath
Principal Executive
Sir Dorabji Tata Centre for Research in Tropical Diseases
Bangalore

C. Durga Rao
Professor and Chairman
Department of Microbiology and Cell Biology
Indian Institute of Science
Bangalore

Sponsored by
Sir Dorabji Tata Trust
Society for Innovation and Development
Indian Institute of Science, Bangalore

On behalf of
Sir Dorabji Tata Centre for Tropical Diseases
In Conjunction with
Department of Microbiology and Cell Biology
Indian Institute of Science, Bangalore

Tata McGraw-Hill Publishing Company Limited
NEW DELHI

McGraw-Hill Offices

New Delhi New York St Louis San Francisco Auckland Bogotá Caracas
Kuala Lumpur Lisbon London Madrid Mexico City Milan Montreal
San Juan Santiago Singapore Sydney Tokyo Toronto

Arthropod Borne Viral Infections—Current Status and Research

Editors

D. Raghunath
Principal Executive
Sir Dorabji Tata Centre for Research in Tropical Diseases
Bangalore

C. Durga Rao
Professor and Chairman
Department of Microbiology and Cell Biology
Indian Institute of Science
Bangalore

Sponsored by
Sir Dorabji Tata Trust
Society for Innovation and Development
Indian Institute of Science, Bangalore

On behalf of
Sir Dorabji Tata Centre for Tropical Diseases
In Conjunction with
Department of Microbiology and Cell Biology
Indian Institute of Science, Bangalore

Tata McGraw-Hill Publishing Company Limited
NEW DELHI

McGraw-Hill Offices

New Delhi New York St Louis San Francisco Auckland Bogotá Caracas
Kuala Lumpur Lisbon London Madrid Mexico City Milan Montreal
San Juan Santiago Singapore Sydney Tokyo Toronto

Arthropod Borne Viral Infections

Current Status and Research